AWARD NUMBER: W81XWH-14-1-0590

TITLE: "A Riboproteomic Platform to Identify Novel Targets for Prostate Cancer Therapy"

PRINCIPAL INVESTIGATOR: Dr. Pier Paolo Pandolfi de Rinaldis

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	29 Sep 2014 - 28 Sep 2015

4. TITLE AND SUBTITLE
A Riboproteomic Platform to Identify Novel Targets for Prostate Cancer Therapy

5a. CONTRACT NUMBER
W81XWH-14-1-0590

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Pier Paolo Pandolfi, MD, PhD

E-Mail: ppandolf@bidmc.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center

330 Brookline Avenue
Boston, MA 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This study aims to take a new approach to studying this process at the level of the translating ribosome and its associated proteins (i.e. the riboproteome). While the conventional wisdom has been that ribosome composition is absolutely fixed, we have been pursuing a line of investigation showing that it is, in fact, flexible and dynamic. Moreover, it has become apparent that the deregulation of the ribosome is implicated in disease initiation and progression, and could serve as a potential target for therapeutic intervention.

Our objective is to analyze the riboproteome in a high-throughput manner in order to gain a global snapshot of all proteins that constitute the riboproteome, to evaluate which of these proteins are altered between different prostate cancer cell lines and types, and to uncover how the riboproteome is altered during prostate cancer development and progression. In addition our studies aim to understand how the riboproteome responds to androgen signaling, and the use of PI3-kinase and MAP-kinase inhibitors, each of which are clinically relevant therapeutic options for prostate cancer patients.

15. SUBJECT TERMS
Prostate cancer, translation, riboproteome, SILAC-based mass spectrometry

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	UU	11	USAMRMC

Table of Contents

1. INTRODUCTION:

In our study, we aim to address the lack of an experimental platform to study the composition of the ribosome and its associated proteins using a mass spectrometry platform to systematically analyze the riboproteome of prostate cancer cells. This methodology has already been optimized in our lab (Reschke et al., 2013), where profound differences in the riboproteomes of normal and cancer cells have been uncovered. These data suggest that the riboproteome and its associated translational landscape are altered during transformation and that significant differences exist between cancer cells. Additionally, we identified various novel proto-oncogenic regulators of translation that have a direct implication for the etiology of prostate cancer including the RNA-binding protein Musashi and the myristoylated alanine-rich C-kinase substrate (MARCKS). Through our studies outlined here we now establish MARCKS to be an important regulatory protein contributing to control of translation in prostate cancer.

2. KEYWORDS:

Prostate cancer, translation, riboproteome, SILAC-based mass spectrometry

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of this project are to establish the importance and relevance of translation and components of the riboproteome for human prostate cancer. In particular our efforts are focused on the following specific goals

Goal 1: To profile and stage prostate cancer riboproteomes using prostate cancer cell lines that represent a spectrum of early to late stage prostate cancer.

Goal 2: To define how androgen signaling and pharmacological inhibition of key signaling pathways impact the riboproteome.

Goal 3: To validate differential components of the riboproteome in primary prostate cancer specimens and patient-derived xenografts.

What was accomplished under these goals?

<u>Major Activities:</u>

The current major activities are focused on (1) development and identification of a robust and comprehensive prostate cancer cell line mix – that will be representative of prostate cancer cell lines, and (2) the further characterization of MARCKS, a riboproteome component that is differentially expressed in prostate cancer, to understand its role and function in the context of translation and prostate cancer development.

In order to further refine our protocol for the characterization of the prostate riboproteome we are preparing a *"prostate specific super-SILAC mix"* that is prepared from polysome fractions isolated from the prostate cell lines, and which will be used as a common standard to compare all our samples to. This approach has previously been shown to be very successful as a way to accurately quantify human breast tumor proteomes (Geiger et al., 2010). To generate this control we are evaluating the following cell lines: PC3, Du145, LnCaP, VCaP, 22Rv1, CA HPV-10, RWPE1, PWR1E, WPE1-NB14, WPE1-NB26.

A label-free MS analysis that enable routine evaluation of more than 3,000 proteins will be performed, followed by the principal component analysis (PCA) of the MaxQuant results. The label-free quantification intensities

(LFQ intensities) will be used for the analysis. The cell lines for the super-SILAC mix will be selected to resemble the experimental systems and represent a broad range of samples. Selected for the super-SILAC mix 5-7 prostate cancer cell lines will be labeled with heavy SILAC media. The use of this reference polysome lysate will allow us to directly compare the riboproteome of all prostate cancer cells and correlate the relative abundance of riboproteomic components between datasets.

The preparation of samples and a label-free mass spectrometry analysis is currently in progress.

Additionally our characterization of the role of the riboproteomic component MARCKS in translation and how it may function in the context prostate cancer has been a major activity of the last period, and we now have strong mechanistic data that establish an important role for this protein in regulating cellular translation.

Significant Results

Having Identified MARCKS as a riboproteomic component we have worked to understand how this protein may function in cellular translation.

To demonstrate that MARCKS was directly associated with ribosomes we next employed a micrococcal nuclease (MN) assay previously described and optimized (Darnell et al., 2011). Treatment of sucrose-gradient purified polyribosomes with MN, followed by separation of ribosomes from released material by ultra-centrifugation, revealed that a significant proportion of MARCKS reproducibly pelleted with ribosomes (Figure 1A, lanes 3 and 5). MARCKS was also observed to be present in the supernatant, even in the absence of MN (Figure 1A, lanes 2 and 4), which is in full agreement with the fact that this protein has additional known functions independent of ribosomal interaction. As expected, MN treatment readily released the RNA binding protein PABP from polyribosomes whereas MN did not affect the ribosomal proteins RpL7a and RpS6 (Figure 1A) (Darnell et al., 2011).

To further confirm the interaction of MARCKS with ribosomes we over-expressed FLAG-tagged MARCKS in HEK293T cells to identify interaction partners by FLAG-immunoprecipitation (IP) followed by mass spectrometry (Figure 1D). MARCKS and several interaction partners were efficiently eluted from the beads using a competitive FLAG peptide when compared to empty vector controls (Figure 1D, lane 4). Strikingly, mass spectrometry analysis revealed a number of ribosomal proteins and ribosome-associated proteins as MARCKS interaction partners (Figure 1C). Furthermore, western blot analysis confirmed that MARCKS co-immunoprecipitated

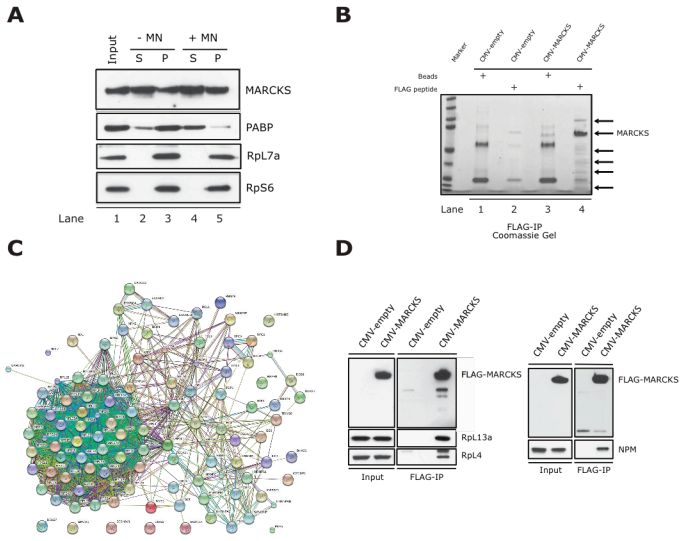

Figure 1. (**A**) Sucrose-gradient purified polyribosomes from PPC1 cells were subjected to micrococcal nuclease (MN, 2000U/ml) treatment to demonstrate that MARCKS is closely associated with ribosomes. Representative western blots for the indicated proteins in the input (collected before centrifugation), the supernatant (S) and the pellet (P) containing ribosome complexes are shown. PABP, RpL7a and RpS6 served as negative and positive controls, respectively. **(B)** Coomassie Gel of FLAG IPs from HEK293T cells overexpression either empty vector or FLAG-MARCKS showing the identification of potential interaction partners of MARCKS (black arrows). **(C)** Network of identified interaction partners of MARCKS by mass spectrometry, highlighting a large node of ribosomal proteins. The network has been generated using the STRING software (*25*) (http://string-db.org). **(D)** FLAG-IP and western blot analysis from HEK293T cells overexpressing either empty vector or FLAG-MARCKS demonstrating that MARCKS co-IPs ribosomal proteins and NPM. Western blots from input and IPs for FLAG, RpL13a, RpL4 and NPM are shown.

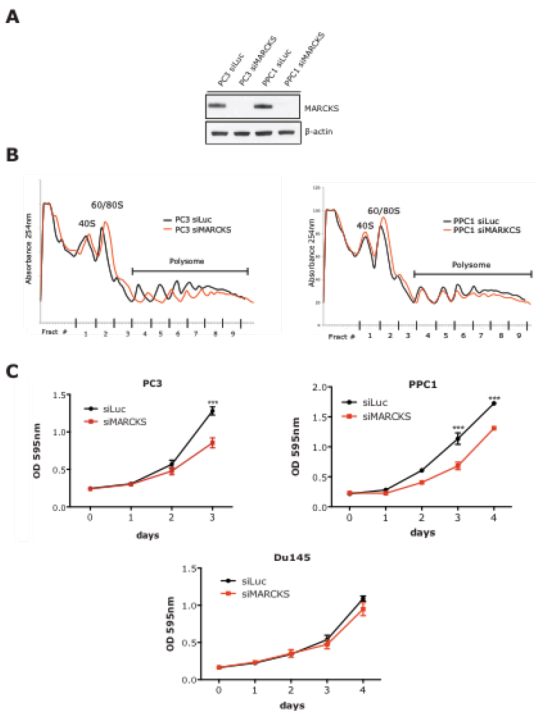

Figure 2. A role for MARCKS in cellular translation. (**A**) Western blot showing MARCKS expression in PC3 and PPC1 cells transfected with siRNA pools against MARCKS. A siRNA against Luciferase (siLuc) served as a negative control and β-actin as control for equal protein loading. (**B**) Polyribosome profile analysis of PC3 and PPC1 cells transfected with either siRNAs against MARCKS or siLuc. Polysome analysis was carried out 72 hours after siRNA transfection. (**C**) Proliferation curve of PC3, PPC1, and Du145 cells treated with siMARCKS or siLuc.

with RpL13a and RpL4-containing complexes as well as with NPM, a protein known to be involved in ribosome biogenesis (Figure 1D) (Grisendi et al., 2006; Maggi et al., 2008). These data further validate the association of MARCKS with the ribosome, and in turn support the hypothesis that MARCKS may contribute to the regulation of cellular translation.

In order to determine the role of MARCKS in tumorigenesis and translation, we used siRNA pools to deplete MARCKS from PC3 and PPC1 prostate cell lines (Figure 2A). Strikingly, suppression of MARCKS resulted in a notable decrease in the amount of polyribosomes in both prostate cancer cell lines when compared to control transfected cells (Figure 2B). In line with the decrease in translational output, we observed an inhibition of cell proliferation in both cell lines upon suppression of MARCKS (Figure 2C). Notably, knockdown of MARCKS in Du145 cells, which contain little or no MARCKS at the polysome, did not result in evident growth suppression (Figure 2C), confirming specificity of the siRNA reagent towards MARCKS.

These data prompted us to consider a role for MARCKS in the regulation of global translation, especially given its strong interaction with the numerous ribosomal proteins described above (Figure 1C and 1D). Intriguingly, we identified eIF2β (*EIF2S2*), a component of the eIF2 translation initiation complex, as part of the MARCKS interactome. Thus, we chose to examine in greater detail the ability of MARCKS to interact and regulate the eIF2 translation initiation complex. To do this, MARCKS was overexpressed in HEK293T cells and immunoprecipitation experiments revealed the protein to interact with both eIF2α and eIF2β (Figure 3A). The eIF2α subunit is phosphorylated upon various stress stimuli, thereby inhibiting its ability to promote translation (Silvera et al., 2010). We thus hypothesized that MARCKS may favor the activity of the eIF2 initiation complex, and in this way contribute to translational regulation. Indeed, western blot analysis from MARCKS overexpressing HEK293T cells demonstrated a clear reduction in the phosphorylation of the eIF2α subunit (Figure 3B, left panel). Consistent with this finding, the knockdown of the MARCKS protein in PC3 cells shows an increase in the phosphorylation of eIF2α (Figure 3B, right panel). These data suggest that MARCKS may help protect cells from stress induced signaling, by directly interacting with the eIF2 translation initiation machinery and blocking inactivation of this complex. To confirm that the effects of MARCKS overexpression on eIF2α phosphorylation actually takes place on the polysome, we next carried out polysome profiling on HEK293T cells transfected with control or MARCKS expressing vector (Figure 3D). Overexpression of MARCKS was seen to promote polysome formation (in contrast to knockdown observations in Figure 3C), with accumulation of ribosomal subunits and a concomitant decrease in 80S monosome (Figure 3D, upper panel). On the other hand, MARCKS knockdown shows a reverse effect on polysome formation (Figure 3C). Western blot analysis on protein isolated from individual fractions demonstrated a clear reduction in the level of phosphorylated eIF2α, while total eIF2α protein levels remain constant when comparing control and MARCKS overexpressing cells, and the cells with knockdown MARCKS levels or control (Figures 3C and D, lower panels).

Therefore, we identified a number of proteins previously not known to be associated with actively translating ribosomes (e.g. MARCKS, Integrin b1 and ICAM1). Within this group, we validate MARCKS as a novel regulator of translation, and a potential biomarker. We show that MARCKS can specifically associate with polyribosomes and that it is required for efficient polyribosome formation and cancer cell proliferation. MARCKS impacts cellular translation through both direct binding to the ribosome, to translation initiation factors and to ribosome-associated proteins. Importantly, we find that through these interactions MARCKS regulates both global and specialized translation. The function of MARCKS in cancer cells, at least in part, may be to maintain active translation in the context of cellular stress, through its ability to interact with the eIF2 translation initiation complex. Interestingly, cellular stress and PKC can regulate MARCKS expression levels (data not shown), and through its ability to regulate the phosphorylation of eIF2a, MARCKS functions to maintain translation under stressful conditions, highlighting its role as an important stabilizer of global translation and its proto-oncogenic potential. This is of particular relevance in oncogenic conditions, which are often accompanied by high levels of intracellular oxidative stress (Sosa et al., 2013).

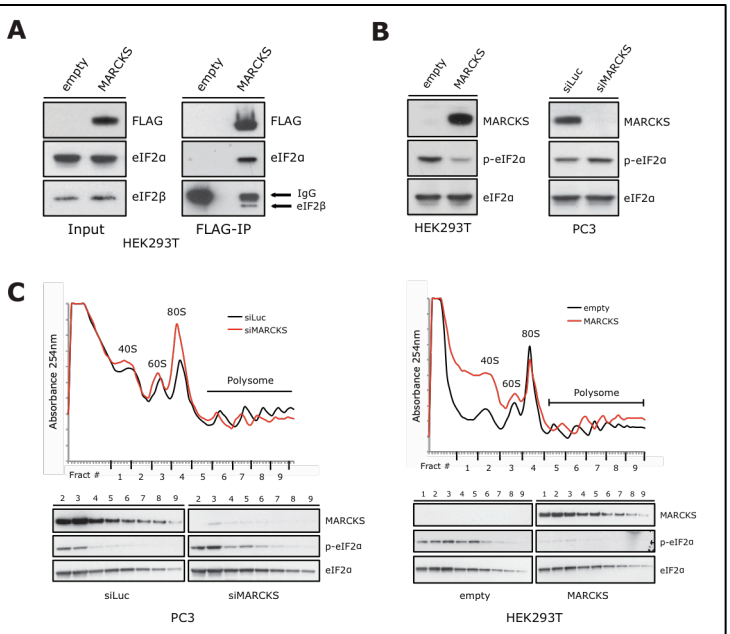

Figure 3. (**A**) FLAG-IP and western blot analysis from HEK293T cells overexpressing either empty vector or FLAG-MARCKS demonstrating that MARCKS co-IPs translational complex proteins eIF2α and eIF2β. (**B**) Western blot analysis from HEK293T cells overexpressing either empty vector or FLAG-MARCKS demonstrating that MARCKS overexpression decreases p-eIF2α expression level. Western blot analysis from PC3 cells transfected with siRNA pools against MARCKS or siRNA against Luciferase (siLuc) shows that MARCKS knock-down increases p-eIF2α expression level. (**C**) Polysome profiling on HEK293T cells transfected with control or siMARCKS pool. (**D**) Polysome profiling on HEK293T cells transfected with control or MARCKS expressing vector. Western blot analysis on protein isolated from individual fractions (lower panels) demonstrates the level of phosphorylated eIF2α, total eIF2α and MARCKS proteins.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

1 – Complete analysis of riboproteomic changes during prostate cancer progression. In order to characterize more specifically the riboproteomic changes that occur during prostate cancer progression *in vitro* we will use a well-characterized panel of tumorigenic cell lines derived from RWPE1 prostatic epithelial cells after exposure to N-methyl-N-nitrosourea (MNU) (these cell lines are commercially available from ATCC). This family of cell lines mimics multiple steps in tumor progression from normal epithelium to PIN to invasive cancer (WPE1- NA22, WPE1-NB14, WPE1-NB11, WPE1-NB26 in order of increasing malignancy) and will allow for a detailed analysis of how the riboproteome changes with increasing malignancy (Webber et al., 2001). Briefly, polysomes will be isolated from RWPE1, WPE1-NA22, WPE1-NB14, WPE1-NB11 and WPE1-NB26 cells and the samples will be mixed 1:1 with the *"prostate specific super-SILAC mix"*. In order to determine how the riboproteome changes during tumor progression we will apply a comprehensive bioinformatics analysis of the datasets obtained in collaboration with Dr. Beck.

2 - Define how androgen signaling and pharmacological inhibition of key signaling pathways impact the riboproteome. Given that prostate riboproteomes display profound changes during tumor development (Reschke et al., 2013) we hypothesize that such changes may dictate the response to androgen and be an important mechanism in the development of resistance to this therapy. To define how androgen affects the composition of the riboproteome we will conduct a SILAC-based riboproteome screen on androgen-sensitive prostate cancer cells (i.e. LnCaP) comparing samples prior to and after androgen stimulation. In addition, as mentioned above, our preliminary data analyzing the riboproteome upon mTOR inhibition (i.e. using the mTOR inhibitor PP242) has identified profound dynamic changes. We will further expand this analysis to encompass the use of PI3-kinase (PI3K) (BKM120, Novartis) and MAP-kinase (MAPK) inhibitors (PD0325901, Pfizer), inhibitors that are currently under pre-clinical and clinical evaluation for prostate cancer therapy. We propose to investigate the consequence and implications of these changes for cellular homeostasis and signaling. These data will have important implications for our understanding of how modulation of riboproteome composition may be utilized to develop novel therapeutic modalities for the treatment of prostate cancer.

3 - Complete validation of differential components of the riboproteome in primary prostate cancer specimens and patient-derived xenografts. Based on the results obtained in Aims 1 and 2, and on preliminary data already collected, we will validate candidate proteins as *bona fide* riboproteome components that are relevant for translation. Additionally, we will clinico-pathologically validate differentially enriched ribosome-associated proteins on primary prostate cancer specimens using immunohistochemistry (IHC) and tissue microarrays (TMA). Based on these data candidates will be further selected that are either strongly over-expressed or lost in human prostate cancer and that significantly correlate with clinical parameters (e.g. overall survival, drug response, increased risk of progression, and androgen sensitivity/resistance). Together, these data will allow us to define androgen, PI3K, and MAPK sensitive markers at the level of the riboproteome. Our tissue microarray will further allow us to potentially link the expression of candidates to specific genetic cancer lesions (e.g. *PTEN* loss, *MYC* amplification, *TMPRSS2* gene fusions) for patient stratification. We will also functionally validate these genes using patient-derived xenografts (PDX) obtained from the Jackson Laboratory.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Deregulation of translational control marks a key event in prostate cancer development and it is well established that the malignancy of cells is strongly linked to and dependent on aberrant protein synthesis. Current knowledge clearly highlights deregulation of protein synthesis, in the development of prostate cancer, through aberrant activation of classical signaling pathways. It has been also hypothesized that aberrant composition of the translational apparatus itself (i.e. the composition of ribosomal and ribosome-associated proteins) can contribute to the transformation process. To date, however, the lack of an experimental platform to study the composition of the ribosome and its associated proteins in a high-throughput and systematic manner has impeded the validation of this hypothesis. Therefore, our research addresses this outstanding issue and provides a robust SILAC-based mass spectrometry platform to systematically analyze the riboproteome of prostate cancer cells.

What was the impact on other disciplines?

These data can have important implications for the role of translation in cancer in general, and may be extrapolated for the benefit and understanding of general mechanisms of translational control in the progression of this disease.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

We decided to perform a label-free evaluation of prostate cell lines for the Super-SILAC Mix followed by the principal component analysis (PCA) of the results. This approach allows us to select the cell lines for the super-SILAC mix that resemble the experimental systems and represent a broad range of samples. Selected for the super-SILAC mix 5-7 prostate cancer cell lines will be labeled with heavy SILAC media. The use of this reference polysome lysate will allow us to directly compare the riboproteome of all prostate cancer cells and correlate the relative abundance of riboproteomic components between datasets.

Actual or anticipated problems or delays and actions or plans to resolve them

We have changed the methodology and planning of SILAC-based mass spectrometry approach but we do not anticipate delay in executing the planned experiments. We also plan to carry out further MARCKS studies following the exciting progress we made in defining its translational role.

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

Nothing to Report

Publications, conference papers, and presentations

Nothing to Report

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Other Products

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Pier Paolo Pandolfi
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.2
Contribution to Project:	Dr. Pandolfi has supervised the work carried out and planned and analyzed experiments
Funding Support:	

Name:	John Clohessy
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.04
Contribution to Project:	Dr. Clohessy has planned and analyzed experimental data and will lead PDX development that will be carried out in Year 2
Funding Support:	

Name:	Yulia Shulga
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	7
Contribution to Project:	Dr. Shulga has performed work in the area of preparation of polysome samples for the mass spectrometry
Funding Support:	Canadian Institutes of Health Research

What other organizations were involved as partners?

Nothing to Report